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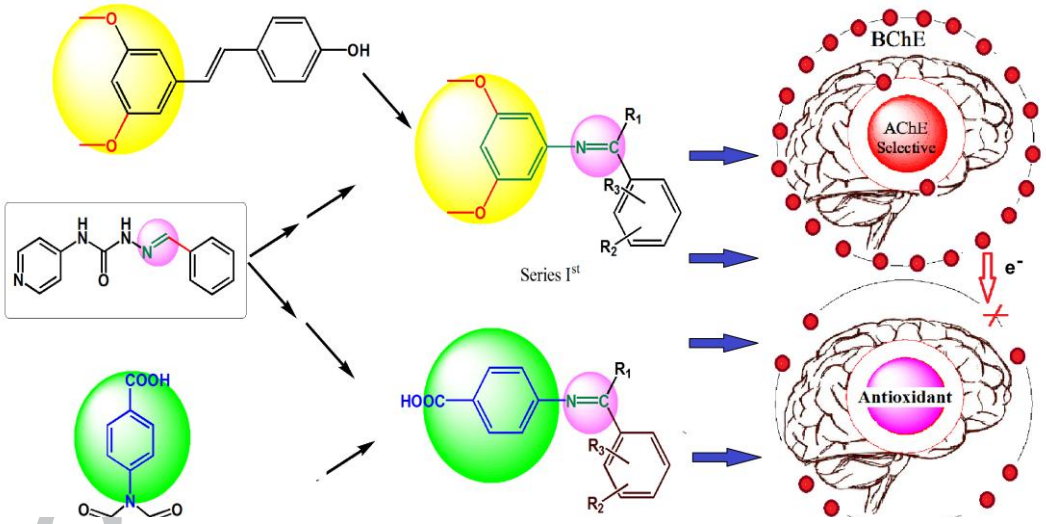

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ABSTRACT

Series of some 3,5-dimethoxy-*N*-methylenebenzenamine and 4-(methyleneamino)benzoic acid derivatives comprising of *N*-methylenebenzenamine nucleus were designed, synthesized, characterized, and assessed for their acetylcholinesterase (AChE), butyrylcholinesterase (BChE) inhibitory, and antioxidant activity thereby improving learning and memory in rats. The IC_{50} values of all the compound along with standard were determined on AChE and BChE enzyme. The free radical scavenging activity was also assessed by *in-vitro* DPPH (2, 2-diphenyl -1-picryl -hydrazyl) and hydrogen peroxide radical scavenging assay. The selective inhibitions of all compounds were observed against AChE in comparison with standard donepezil. The enzyme kinetic study of the most active compound **4** indicated uncompetitive AChE inhibition. The docking studies of compound **4** exhibited the worthy interaction on active-site gorge residues Phe330 and Trp279 responsible for its high affinity towards AChE, whereas lacking of the BChE inhibition was observed due to a wider gorge binding site and absence of important aromatic amino acids interactions. The *ex vivo* study confirmed AChE inhibition abilities of compound **4** at brain site. Further, a considerable decrease in escape latency period of the compound was observed in comparison with standard donepezil through *in-vivo* Spatial Reference Memory (SRM) and Spatial Working Memory (SWM) models which showed the cognition-enhancing potential of compound **4**. The *in-vivo* reduced glutathione (GSH) estimation on rat brain tissue homogenate was also performed to evaluate free radical scavenging activity substantiated the antioxidant activity in learning and memory.

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1. Introduction

The acetylcholine (ACh) plays a significant role in strengthening of synaptogenesis[1] of active neurons for up-regulating the memory and learning in humans.[2] It is not only parasympathetic neurotransmitter but also act as the memory enhancer.[3] Acetylcholinesterase (AChE) is a cholinesterase enzyme that breaks the ACh, whereas inhibitors of AChE impede the breakdown of acetylcholine (ACh) and improves the neuronal acetylcholine amount. The crystal structure of AChE suggested that its active site possessed a 20 Å deep gorge. Initially, the binding of Ach at acetylcholinesterase enzyme occurs at the outer gorge, known as the peripheral anionic site (PAS) lined with Trp279, Tyr70, Tyr121, and Phe290. The bottom of the gorge containing four main subsites, (i) the esteratic site, (ii) the oxyanion hole, (iii) the anionic subsite and, (iv) the acyl pocket, where the hydrolysis of Ach takes place.[4] The butyrylcholinesterase (BChE), a non-specific cholinesterase enzyme found in both plasma and brain and capable of hydrolyzing ACh and the other esters. The BChE also get inhibited by the non-specific AChE inhibitors, leads to hepatotoxicity and other side effects.[5] Moreover, the primary structural difference between AChE and BChE is the absence of the PAS moiety in BChE at the top of the gorge; hence, it seems clear that an inhibitor of cholinesterase, exhibiting strong interaction with the PAS would have greater AChE specificity over BChE.[4] The kinetics of such inhibitors are reported as reversible competitive, non-competitive, mixed and un-competitive inhibition.[6]

The deficits of working and reference memory due to the lack of AChE are considered as a feature of many neuroinflammatory disorders.[7] These short-term (working) memory and long-term (reference) memories are ushered to learn well under cognition and behavioral studies.[8(a,b)]

In search of a novel drug, Schiff base (azomethine -C=N- functional group) have been attracting scaffold because of its various therapeutic applications in drug discovery such as antioxidant,[9] anti-Alzheimer,[10] antituberculosis,[11] anticancer,[12] antiepileptic,[13] etc.

The Schiff bases of *p*-aminobenzoic acid are reported to possess a high tendency to form metal chelate complexes that elicit a plethora of pharmacological activities. [14(a-e)] Several amide- and imide derivatives of *m*- and *p*-aminobenzoic acid have been synthesized and evaluated for their AChE activity.[15] Pyrrolo-isoxazole derivatives are also mentioned in literature as potent anticholinesterase and anti-amnesic agents. In our previous studies, various Schiff bases of 4-aminopyridine[16] and semicarbazone[17] were synthesized and evaluated for AChE inhibitory activity. The Schiff bases of *p*-aminobenzoic acid have been reported for various pharmacological activities, but it had never been studied for selective AChE inhibition in cognitive dysfunction. Schiff bases could be a promising pharmacophore to treat the memory and learning.

Currently, some Schiff-bases have been investigated as effective scavengers of Reactive Oxygen Species (ROS) that acts as an antioxidant. The free radical scavenging effects of the Schiff base may be due to its imino group which extends free radical delocalization.[18] Also, the nitrogen of the azomethine group (>C=N) in Schiff bases acts as a good donor of the electron due to the availability of loan pair of electrons on the nitrogen atom. The electron donating character of the double bond, and low electronegativity of nitrogen makes Schiff base an active ligand for antioxidant activity.[19]

Recent studies proved that increased oxidative stress (an imbalance between the generation of free radicals and the ability of the body to counterattack or detoxify their damaging effects) is one of the causes of impaired learning behavior that modestly reduces the motor activity.[20] Hence, the compounds like Schiff-base having both AChE as well as free radical scavenging activity could be more effective in the study of learning and memory.

Natural products polyhydroxy stilbenes like pterostilbene have been extensively exploited for anticancer, anti-inflammatory, neuroprotective, antioxidant and AChE inhibitory activities with low neurotoxicity that inferred the potential tropism of polyhydroxy stilbene in uplifting learning and memory.[21(a,b)] Recent patent suggests that pterostilbene is effectively improvise working memory and reversing memory deficit.[22] The isosteric replacement of ethenyl carbon of stilbene with nitrogen shows cation-π stacking interaction with the AChE Trp-84 at anionic subsite.[23] This imine also incorporates the required features for metal chelation and antioxidant activities as stated in the literature. The Schiff bases of polymethoxy stilbenes are reported as novel intermediate for heterocyclic's (indazoles) and suggest to be a promising lead scaffold for Rational Drug Design of AChE inhibitors.[24(a,b)]

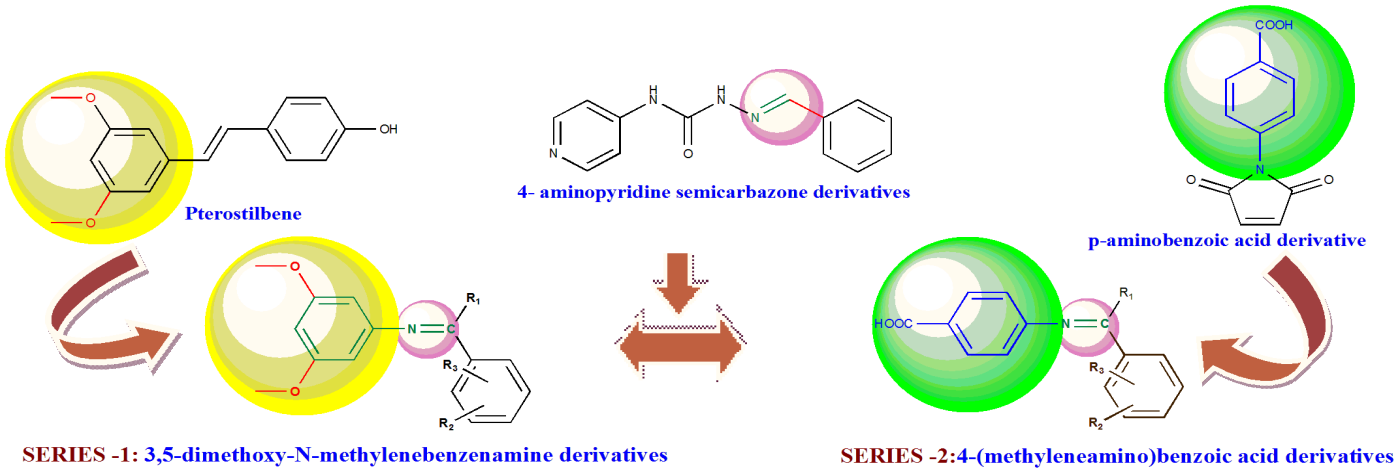
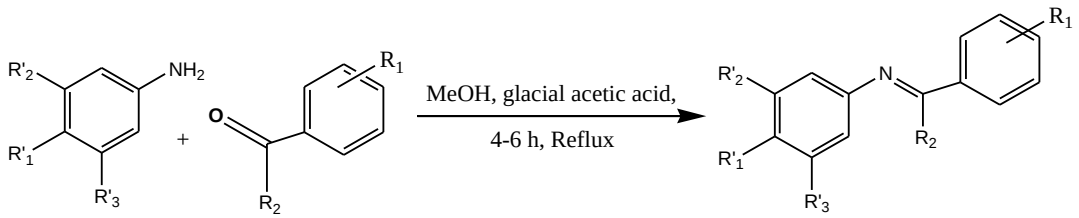


Figure 1. The Design of compounds as potential agents to enhance learning and memory (Series-1) 3,5-dimethoxy-N-Methylenebenzenamine derivatives (Series-2) 4-(methyleneamino)benzoic acid derivatives.



Scheme 1

Table 1. Synthesized *N*-Methylenebenzenamine derivative

Compound Code	R'$_1$	R'$_2$	R'$_3$	R$_1$	R$_2$	Yield (%)	Melting Point (°C)	R$_f$*
Series 1: 3,5-dimethoxy-*N*-Methylenebenzenamine derivative								
1[a]	H	OCH_3	OCH_3	2-OH	H	79	92-94	0.84
2	H	OCH_3	OCH_3	4-NO_2	H	72	84-86	0.85
3	H	OCH_3	OCH_3	4-OCH_3	C_6H_5	76	115-117	0.89
4	H	OCH_3	OCH_3	3,4,5-tri OCH_3	H	55	79-81	0.82
5[b]	H	OCH_3	OCH_3	2- NO_2	H	59	122-124	0.83
Series 2: 4-(methyleneamino)benzoic acid derivative								
6[c]	COOH	H	H	2- OH	H	84	276-278	0.81
7[d]	COOH	H	H	H	H	79	121-123	0.78
8[e]	COOH	H	H	2- NO_2	H	77	220-222	0.80
9[f]	COOH	H	H	4- NO_2	H	75	180-182	0.86
10[g]	COOH	H	H	3,4,5-tri OCH_3	H	71	180-182	0.90
11	COOH	H	H	4-OCH_3	C_6H_5	74	194-196	0.87
12	COOH	H	H	4-OH, 3-OMe	CH_3	71	210-212	0.74

Reference; 1[a]: 11a, 5 [b]: 11b, 6[c]: 12a, 7[d]: 12b, 8 [e]: 12c, 9 [f]: 12d, 10[g]: 12e, *: Solvent system: Chloroform / methanol (4.5:0.5)

In this paper, we have designed and synthesized some lipophilic 3,5-dimethoxy-*N*-methylenebenzenamine and 4-(methyleneamino)benzoic acid derivatives having the *N*-methylenebenzenamine nucleus (Figure 1) and evaluated for the *in-vitro* AChE and BChE inhibitory activity. The enzyme kinetic study was performed to assess its type of inhibition. Further, the most active compound **4** was assessed for the *in-silico* docking analysis followed by *ex vivo* studies of AChE inhibition and the *in-vivo* Spatial Reference Memory (SRM), Spatial Working Memory (SWM) evaluation. We have also performed an *in-vitro* DPPH (2, 2-diphenyl -1-picryl -hydrazyl) and hydrogen peroxide radical scavenging assay for free radical scavenging activity further this study was supported by *in-vivo* reduced glutathione estimation.

2. Results and discussion

2.1. Chemistry

The compounds were synthesized by the nucleophilic addition of the amine to the carbonyl group forming an unstable aminomethanol intermediate followed by dehydration in an acidic environment to generate an imine (Scheme 1 and Figure 1 of supporting information).[25] Basically, in this synthetic condition the 3,5-dimethoxyaniline and p-aminobenzoic acid (PABA) acted as the nucleophile for various carbonyl compounds as listed in Table 1. Preliminary identification of imine was confirmed by positive Dragendroff test on TLC (Thin layer chromatography). The purity of the compounds was checked and verified by state of the art spectroscopic technique and elemental analysis. The FT-IR of all the compounds showed a diagnostic stretching vibration of (-C=N) imine group in the range of 1578-1610 cm^{-1}. ^1H NMR spectra of all compounds showed a characteristic sharp singlet peak of imine (N=CH) approximately at 8.34 δ value (except compound **3, 11** and **12**). Lead compound **4** showed characteristic singlet peak of the 3,4,5-trimethoxy group at 3.93 δ of nine protons and singlet peak of the 3,5 dimethoxy group at 3.82 δ of six protons. In second series compounds showed a carboxylate peak near 12 δ value having integration value of 1 as a singlet at down field.

2.2. Cholinesterase Inhibition

The strength to inhibit AChE and BChE was observed through their IC_{50} values determination using Ellman method of all synthesized compounds (series 1 & 2) and standard donepezil.

The designed compounds (**1-12**) were initially expected to inhibit both AChE & BChE with the same potential. However, the results of the Ellman test showed that compounds of series 1 & 2 were the selective inhibitor of electric eel AChE having IC_{50} value in the micromole (0.82 to 25.53 µM) over BChE IC_{50} value in the millimole (0.21 to 0.83 mM). The compound **4** (Figure 2) bearing 3,4,5 tri-methoxy group on aldehydic phenyl ring showed a more potent AChE inhibition over synthesized compounds of both series 1 & 2. The non-polar group (OCH_3) has modulated the AChE inhibitory potency due to increased lipophilic characteristic.

All 4-(methyleneamino) benzoic acid derivatives of series 2 possessed a moderate inhibitory activity towards AChE owing to the polar group (-COOH) of benzoic acid as the common scaffold. The 3,5-dimethoxy-*N*-methylenebenzenamine derivative of series 1 having bulky 3,5-dimethoxy group on phenyl ring that played a significant role in the structural-activity

Table 2. IC_{50} values of the synthesized derivatives

Compound code	AChE IC_{50} (µM) ± SEM	BChE IC_{50} (µM) ± SEM	[a]Selectivity Index
Donepezil *	0.04 ± 0.01	15.24 ± 0.88	381 ± 6.33
Series 1			
1	25.53 ± 1.12	746.7 ± 37.27	29.25 ± 1.12[a,b]
2	7.01 ± 0.34	452.4 ± 3.04	64.52 ± 2.23[a,b]
3	4.37 ± 0.23	284.2 ± 10.96	65.06 ± 2.78[a,b]
4**	0.82 ± 0.05	328.0 ± 1.53	401.96 ± 9.45[a]
5	14.35 ± 0.71	456.7 ± 9.17	31.83 ± 1.10[a,b]
Series 2			
6	18.03 ± 0.92	717.2 ± 5.88	39.77 ± 1.31[a,b]
7	12.64 ± 0.59	502.1 ± 1.66	39.71 ± 1.22[a,b]
8	6.00 ± 0.24	386.9 ± 1.94	64.46 ± 2.12[a,b]
9	5.95 ± 0.27	217.1 ± 8.38	36.48 ± 1.88[a,b]
10	11.55 ± 0.42	378.2 ± 19.10	32.73 ± 1.12[a,b]
11	6.09 ± 0.29	291.5 ± 2.20	47.85 ± 1.92[a,b]
12	3.93 ± 0.17	839.0 ± 1.53	213.70 ± 7.55[a,b]

*:Donepezil, ** compound **4**. Values are expressed in the mean ± SEM (n=6), [a]p< 0.05 and [b]p< 0.05 as compared to the donepezil and compound **4**, (one way ANOVA followed by Bonferronipost tests).
[a]Selectivity for AChE is defined as IC_{50}(BChE)/IC_{50}(AChE).[26]

relationship. The presence of methoxy group modulated the AChE inhibitory potency due to the high lipophilic (log P) characteristic of the molecule. The selectivity index of all synthesized compounds was calculated and mentioned in Table 2. The compound **4** showed a significant selectivity index (401.96 ± 9.45) in comparison to standard donepezil (381 ± 6.33).

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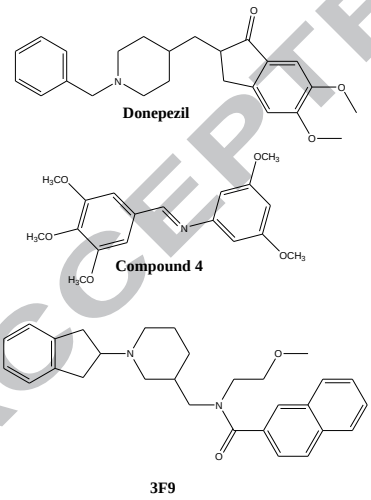

Figure 2. *Chemical structure of donepezil, Compound **4**, and 3F9*

2.3. Enzyme kinetics studies

Further, the compound **4** was subjected to enzyme kinetics study. Lineweaver–Burk plot of compound **4** resulted in a line parallel to the original enzyme-substrate plot, with a higher y-intercept. This characteristic pattern indicated an uncompetitive inhibition of AChE enzyme (Ki = 0.72 ± 0.06 µM) (Figure 3), due to the possible interaction of methoxy group (OCH_3) at the peripheral anionic site of AChE.

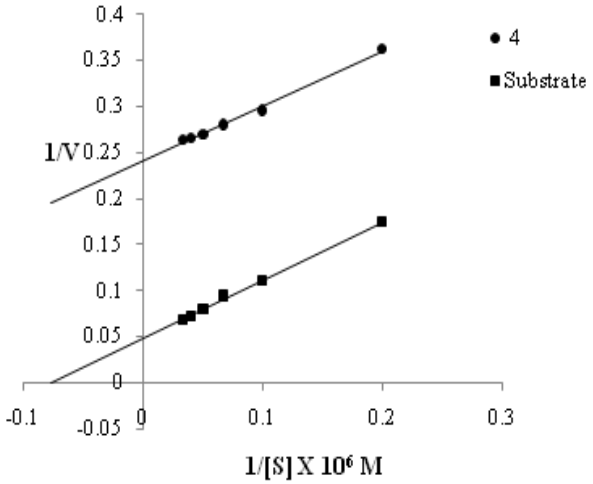

Figure 3. Lineweaver-Burk plot resulting from substrate [S]–velocity [V] curves of AChE activity for compound **4**

2.4. Molecular docking

To understand the cholinesterase inhibition profile, the docking was done on AChE complexed with E2020 (donepezil) (PDB Code: 1EVE) and BChE complexed with N- [(3R)-1-(2,3-dihydro-1H-inden-2-yl)piperidin-3-yl]methyl-N-(2methoxyethyl)naphthalene- 2-carboxamide (3F9) (PDB Code: 4TPK) (Maestro 10.5.014, Glide, Schrödinger, 2016-1)(Figure 2) to provide a better interpretation of the biological profile of compound **4** and donepezil toward enzymes. Validation of docking protocol was done by measuring the root mean square deviation (RMSD) between the actual pose and predicted pose of ligand to the protein. The RMSD of the reported protocol was found to be 0.68, and 1.2868 which fell within the acceptable limit of 2.0 Å for AChE and BChE respectively.

Superposition of the best pose of compound **4** and donepezil at the active site revealed that the binding mode of compound **4** at the PAS region of AChE resembled very much to that of donepezil. In donepezil, residue Trp84 was interacting with ligand *via* π–π stacking interaction at the bottom, while Phe330 and Trp279 were interacting with the ligand through cation–π and π–π stacking interaction at the midpoint and the entrance of the gorge respectively (Figure 4A).The most active compound **4**, which has a Glide score of -7.7 kcal/mol exhibited hydrophobic interaction at PAS resulting in strong binding affinity. The binding affinity was observed through the aromatic ring of the 3,5-dimethoxy ring formed π–π parallel Edge to Face stacking interactions with Phe330, and the tri-methoxybenzene aromatic ring formed π–π Face to Face stacking interactions with Tyr279 (Figure 4B).

Superposition of the best pose of compound **4** and 3F9 was observed at the active site of BChE to gain insight on their high selectivity for AChE over BChE. In 3F9, a strong cation–π stacking interaction was observed between the nitrogen of the piperidine and Tyr332, while the naphthalene moiety was fully occupied the acyl-binding pocket where it stacks to the Trp231 via π–π stacking interaction. The docking of compound **4** on 4TPK (score -4.8 kcal/mol) was not shown any hydrophobic interaction rather exhibited electrostatic interactions at the active site resulted in its weak binding affinity. However, the tri-methoxybenzene aromatic ring formed π–π Edge to Face stacking interactions with Trp82 (Figure 4C). All these results suggested that compound **4** was unable to bind efficiently owing

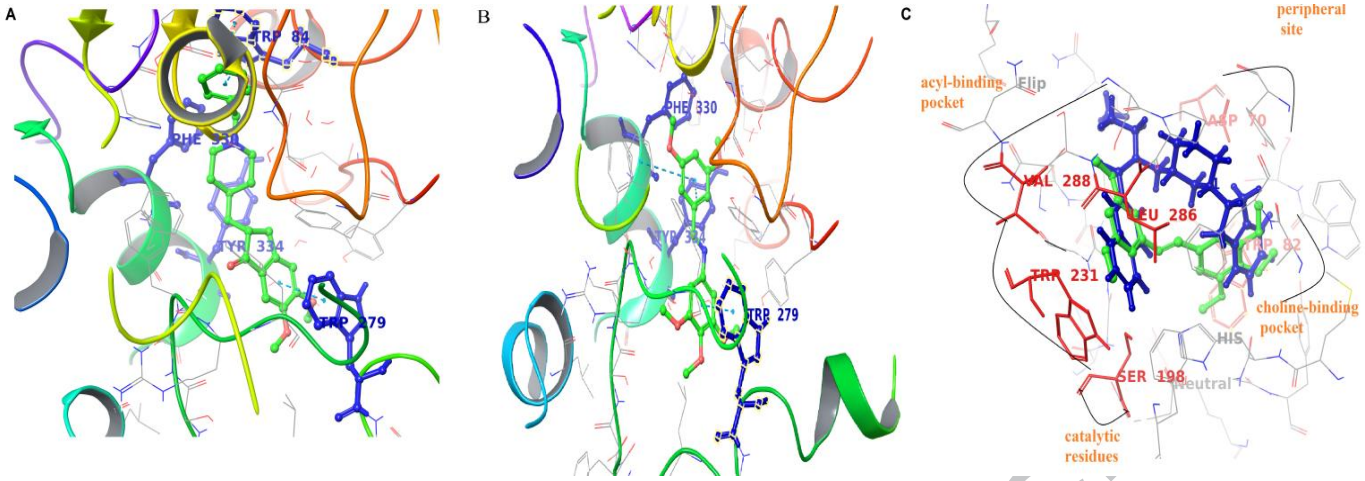

Figure 4. (A) Docking model of donepezil at the active sites of AChE. (B) Docking model of compound **4** at the active sites of AChE. The interactions are shown in green. (C) Superimposition docking model of 3F9 and compound **4** at the active sites of at the active sites of BChE.

Table 3. QikProp Analysis

Compound	CNS[a]	SASA[b]	QPlogBB[c]	QPlogPo/w[d]	QPPCaco[e]	QPPMDCK[f]	QPlogKhsa[g]
4	1	563.19	-0.088	3.82	6411.31	3686.07	0.04
Donepezil	1	714.49	0.11	4.44	884.31	479.198	0.618

[a]CNS - This exhibits the predicted central nervous system activity.(–2 to +2)
[b]SASA- Total solvent accessible surface area (SASA) insquare angstroms using a probe with a 1.4 Å Radius. (300 to 1000)
[c] QPlogBB - Predicted brain/blood partition coefficient. Note: QikProp predictions are for orally delivered drugs so, for example, dopamine and serotonin are CNS negative because they are too polar to cross the blood-brain barrier (-3 to +1.2)
[d]QPlogPo/w - This gives the predicted octanol/water partition coefficient. (-2 to 6.5)
[e]QPPCaco - This gives the predicted apparent Caco-2 cell permeability in nm/sec. Caco-2 cells are a model for the gut-blood barrier. (<25 is considered poor and >500 is considered excellent)
[f]QPPMDCK- Predicted apparent MDCK cell permeability in nm/sec using the Affymax scale. (<25 is considered poor and >500 is considered excellent)
[g]QPLog Khsa -Prediction of binding to human serum albumin. Predictions are for non-active transport. (–1.5 to 1.5)

to the (i) small molecular size of compound **4** relative to the larger volume of the bottom gorge, good to accommodate bulkier substrate. (ii) no major interaction at the peripheral site, choline binding pocket, and catalytic site. (iii) lack of aromatic amino acid at the bottom of the gorge for hydrophobic interaction.

This interaction study demonstrated that compound **4** strongly bound to the binding PAS sites of AChE, supporting the potential inhibitory activity of compound **4** which was supported by the kinetic study exhibiting binding of compound **4** to the PAS sites.

2.5. Log P determination and in silico pharmacokinetic parameters

It is a well-known fact that lipophilicity (log P) is an important physicochemical parameter and it has the strong relationship with *in-vivo* brain penetration of drugs. Compounds possess moderate lipophilicity (lower than 4) often exhibit the highest uptake in the brain.[27] Other physicochemical parameters e.g. Caco-2 cell permeability (PPCaco), brain/blood partition coefficient (log BB), and MDCK cell permeability (PPMDCK) are also the indicators of the lipophilicity of the molecule.[28] We have determined the partition coefficient of all the synthesized compounds and standard by octanol/water system (Supplementary Table 1) and QlogBB, QlogPo/w, QPPCaco, QlogKhsa, and QPPMDCK were calculated through QikProp software tool (Table 3). QikProp analysis resulted in an accurate, quick, and comprehensive, prediction of adsorption, distribution, metabolism, and excretion. The *in-silico* ADME/Tox Predictions were analyzed through QikProp (Maestro 10.5.014, Schrödinger, LLC, and New York-1). The result showed that compound 4 had a better log P value (3.27) than standard donepezil (4.24) and considered optimal to cross the blood-brain barrier.

The combined result of *in-vitro*, and *in-silico* studies indicated that compound **4** could be a good candidate for *ex vivo* and *in-vivo* assessment of learning and memory.

2.6. Free radical scavenging activity

Recent studies proved that increased oxidative stress causes impaired learning behavior and reduces motor activity. It is also reported that the antioxidants and vitamin-rich diet could improve the cerebellar physiology and motor learning of aged rats and also improve the performance of learning in rat on water maze model at the lower dose.[29]

The free radical scavenging activity of the synthesized compound 4 was assessed by established *in-vitro* DPPH and hydrogen peroxide methods. The *in-vitro* activity was performed to determined the scavenging capability through DPPH (2,2-diphenyl-1-picryl-hydrazyl)[30] and Hydrogen peroxide radical scavenging activity.[31] The *in-vitro* results showed that compound **4** have better antioxidant activity compared to donepezil in both assays. The result is summarized in Figure 5.

2.7. Biological Evaluation

2.7.1. Ex vivo AChE inhibition

Ex vivo cholinesterase inhibition was determined by Ellman method. The result showed that the compound **4** significantly inhibited AChE activity in the brain compared to that of control (Table 4). These results confirmed the ability of compound **4** to cross the blood brain barrier.

Table 4. Effect of compound **4** on acetylcholinesterase activity in brain regions of the rat

Treatment [Dose(mg/kg)]	'*n*' moles of substrate hydrolyzed/min/mg protein
Control	46.56 ± 0.66
Compound **4** (10.0)	33.78 ± 0.94*
Compound **4** (30.0)	30.85 ± 0.47*
Donepezil (10.0)	28.24 ± 0.76*

Data are expressed as means± SEM (n = 3). Data were statistically analyzed by one-way ANOVA. *p < 0.05 compared to the control.

2.7.2 In-vivo Evaluation

2.7.2.1. Learning and memory

The most active compound **4** was further evaluated for Spatial Reference Memory (SRM) and Spatial Working Memory (SWM) and observations were expressed as mean ± SEM. Spatial reference memory data analyzed by repeated measure two-way ANOVA revealed that there were significant differences among treatment [F (4, 60) = 199.4, p < 0.05] and time [F (2, 60) = 1759, p < 0.05]. Post-hoc analysis showed that there was no significant difference among the groups on day one. Further, there was a significant interaction [F (8, 60) = 55.73, p < 0.05] between treatment and time. However, on day two, a considerable decrease was observed in escape latency in donepezil and compound **4** (20 and 30 mg/Kg; p.o.) treated groups compared to the control. There was no significant difference between compound **4**, 20mg/Kg; p.o. and, 30mg/Kg; p.o. treated groups (Figure 6A). To test whether this memory trace was retained and consolidated for future navigation, the probe trial was repeated after the twenty-four hours spatial reference memory task. One-way ANOVA revealed significant differences [F (4, 24) = 17. 14, p < 0.05] among the groups in the probe trial (Figure 6B). Post-hoc analysis indicated that the mean time spent in the target quadrant of the maze by rats treated with compound **4** and donepezil was significantly longer than the control group. A significant time spent in the target quadrant during the probe trial indicated the retention of spatial memory. For the test of spatial working memory, the rat was expected to find a platform placed in a new position during the first trial (acquisition), whereas in the retrieval trial, the platform was kept in its previous position. Statistical analysis using repeated measure two-way ANOVA revealed that there were significant differences among treatment [F (4, 100) = 10.94, p < 0.05] and time [F (3, 100) = 51.76, p < 0.05]. Further there was a significant interaction [F (12, 100) = 2.458, p < 0.05] between treatment and time (Table 5). Post-hoc analysis showed that there was no significant interaction among groups on day one but on succeeding days the most active compound **4** (10, 20 and 30mg/Kg; p.o.) and donepezil (10 mg/Kg; p.o.) showed significant differences in the escape latency compared to the control. However, there was no decrease in escape latency between the doses tested. Similarly, analysis of the retrieval trial data revealed significant differences among treatment [F (4, 100) = 45.15, p < 0.05] and time [F (3, 100) = 77.84, p < 0.05]. Further, there was a significant interaction [F (12, 100) = 3.220, p < 0.05] between treatment and time. Post-hoc analysis revealed that on day one, only the donepezil-treated group showed reduced escape latency compared to the control. On days two, three and four, the compound **4** (10, 20 and 30 mg/Kg; p.o.) treated groups, and the donepezil-treated group showed reduced escape latency than the control. However, there was a greater decrease in escape latency in compound **4** (20 and 30 mg/Kg; p.o.) treated groups compared to compound **4** (10 mg/Kg; p.o.) treated group.

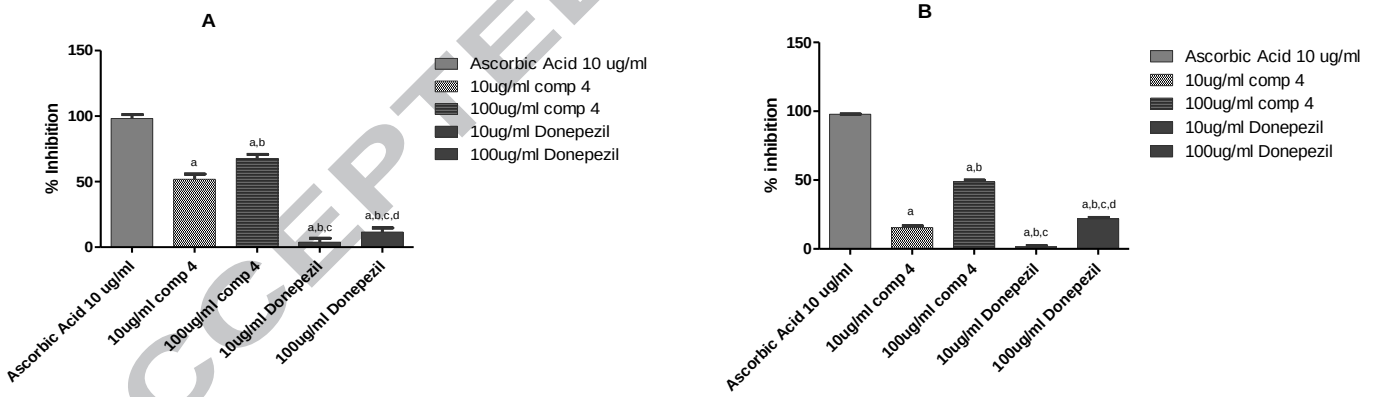

Figure 5. Graphical comparison of the *in-vitro* antioxidant effect of compound **4** and Donepezil **(A)** DPPH assay (**B**) H_2O_2 free radical scavenging assay. Values are expressed as the mean ± SEM (n=3). [a]p< 0.05, [b]p < 0.05 [c]p< 0.05 and [d]p < 0.05 compared to the control, compound **4** (10µg/ml), compound **4** (100µg/ml) and donepezil (10µg/ml) respectively (one-way ANOVA followed by Newman-Keuls test).

Table 5. Nootropic effect of Compound **4** in rats on 'escape latency' in Acquisition phase of SWM

Treatment	Escape Latency (s)			
Dose mg/Kg; p.o.	Day 1	Day 2	Day 3	Day 4
Control	17.45 ± 0.62	16.97 ± 0.80	17.38 ± 0.79	14.12 ± 0.78
Donepezil	16.88 ± 0.61	14.38 ± 0.41[a]	7.78 ± 0.27[a]	7.33 ± 0.31[a]
10mg/Kg 4	17.57 ± 0.56	15.45 ± 0.38[a]	15.12 ± 0.80[a,b]	10.20 ± 0.50[a,b]
20mg/Kg 4	16.95 ± 0.73	15.69 ± 0.64[a]	12.23 ± 0.63[a,b,c]	8.87 ± 0.48[a]
30mg/Kg 4	16.87 ± 0.70	15.23 ± 0.99[a]	10.37 ± 0.46[a,b,c]	8.92 ± 0.43[a]

Values are expressed in the mean ± SEM (n=6), [a]p< 0.05, [b]p< 0.05 and [c]p< 0.05, as compared to the control, donepezil and compound **4**, (dose- 10mg/Kgp.o) respectively (two way ANOVA followed by Bonferronipost tests).

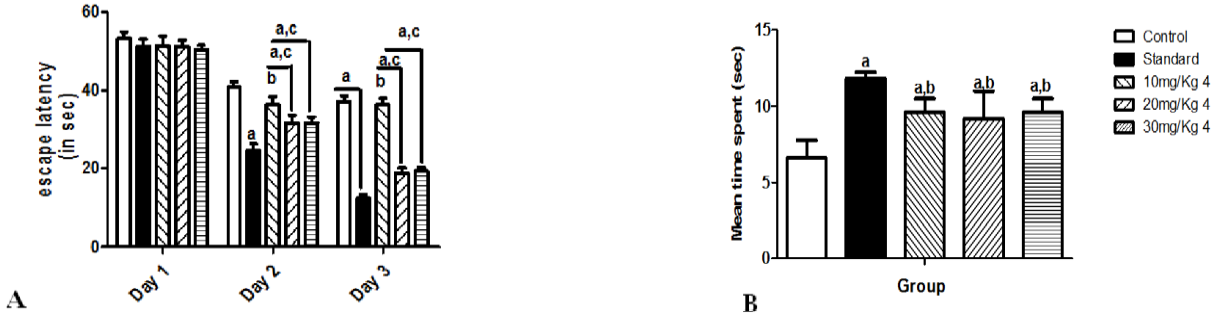

Figure 6. Graphical comparison of nootropic effect **(A)** in terms of 'the escape latency' among various groups in SRM test days 1-3. Values are expressed as the mean ± SEM (n=6). [a]p< 0.05, [b]p < 0.05 and [c]p < 0.05 compared to the control, donepezil and compound **4** , dose 1 (10mg/Kg p.o) respectively (two-way ANOVA followed by Bonferroni post-tests). (**B**) in terms of 'the mean time spent in the target quadrant' among various groups in 'Probe Trial' on day 4. Values are expressed as the mean ± SEM (n=6). [a]p< 0.05 and [b]p < 0.05 compared to the control and donepezil respectively (one-way ANOVA followed by Newman-Keuls test).

An overall positive effect of compound **4** was found consistently throughout behavioral tests where it showed significantly attenuated disruption of long-term spatial navigation in both the SRM and SWM assays. Escape latency was found same on the first day in all the groups including control and standard but a significant difference observed on second day onwards. The physicochemical parameters and appropriate blood-brain barrier permeability reasons for its comparable drug efficacy on day 2nd in the Spatial Reference Memory (SRM) and Spatial Working Memory (SWM) of behavior test. The results suggested that the escape latency of the compounds **4** treated group was significantly ($p<0.05$) more than that of the control .indicating an increased efficacy to the learning and memory abilities. In conclusion, the compound 4 was found to be a promising active molecule for uplifting the learning and memory in rats.

2.7.2.2. Reduced GSH estimation

GSH is a low molecular weight tripeptide which plays a crucial role in counterattacking ROS.[32] Severe depletion of GSH level in the brain due to the oral administration of aspartame was observed.[33] The method illustrated by Ellman[34] was used for determination of antioxidant activity. Moderate increase in GSH level was evident among groups treated with compound **4** (24.52 ±0.26 GSH (µg/mg protein)) compared to control, reflecting its antioxidant nature, whereas the standard donepezil-treated group, the GSH level was found 19.12±1.40 GSH (µg/mg protein), which was insignificant compared to control.(Figure 7)

3. Conclusion

The methoxy-substituted compound 4 demonstrated potent cognition-enhancing. In the docking studies, compound 4 confirmed its interaction with the important active-site gorge residues Phe 330 and Trp279 responsible for its high affinity towards AChE. The compound 4 showed a significant selectivity index in comparison to standard donepezil in *in-vitro* studies. The *ex vivo* study also confirmed the ability of compound 4 to cross the blood brain barrier and selective AChE inhibition at respective brain site owing to its appropriate physicochemical parameter e.g. partition coefficient (log P), Caco-2 cell permeability (PPCaco), brain/blood partition coefficient (log BB), and MDCK cell permeability (PPMDCK), that elicited a considerable decrease in escape latency in SRM and SWM *in-vivo* models. Further, the *in-vitro* DPPH (2, 2-diphenyl -1-picryl -hydrazyl) and hydrogen peroxide radical scavenging assay and *in-vivo* reduced glutathione estimation supported that compound **4** would be a promising drug candidate to treat the learning and memory disorders due to its antioxidant and selective AChE inhibition activity.

In conclusion, from the above studies, we have successfully identified a new class of potent cognition-enhancing chemical entity. Among the identified compounds, compound **4** deserves further studies which can lead to a discovery of a new lead having a potent cognition-enhancing property.

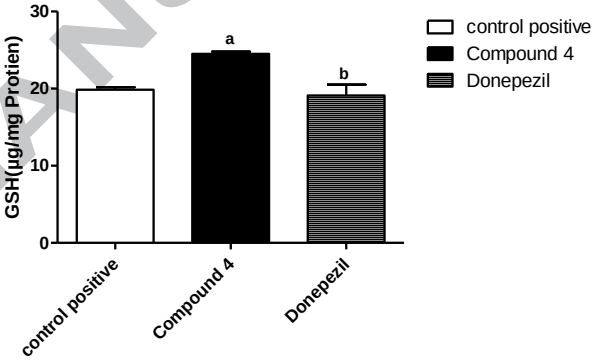

Figure 7. Graphical comparison of *in-vivo* Reduced GSH estimation.of compound **4** and Donepezil. Values are expressed as the mean ± SEM (n=3). [a]p< 0.05 and [b]p < 0.05 compared to the control and compound **4** respectively (one-way ANOVA followed by Newman-Keuls test).

4. Material and methods

4.1. General

All reagents and solvents used in this study were of analytical grade purity and were procured from Sigma-Aldrich (India). Donepezil was obtained as a gift from Cipla Ltd. (Maharashtra, India). The melting points of the compounds were determined in open capillary tubes using a BI 9300 Bumstead/Electrothermal Stuart (SMPIO) melting point apparatus and are uncorrected. The reaction progression was monitored by thin layer chromatography with ethyl acetate: hexane (3:7) as the mobile phase on TLC silica gel 60 F254 aluminum sheets (Merck, India). UV spectral analysis was performed using a JASCO (Model 7800) UV-VIS spectrophotometer. FT-IR spectra were recorded on a Shimadzu FT-IR 8400S spectrophotometer at the scanning range 400–4000 cm^{-1}. ^1H spectra were recorded on a JEOL AL 300 FT-NMR spectrometer in DMSO-d_6. Tetramethylsilane (TMS) was used as an internal standard. Chemical shift values were expressed in parts per million (δ). The purity of the final compounds (>95%) was determined by elemental analysis. Elemental analysis was performed using an Exeter CE-440 elemental analyzer.

4.2. Chemistry

*4.2.1. General procedure for the synthesis of **1-12***

0.1M of para-aminobenzoic acid (Series 1) or 3,5-dimethoxyaniline (Series 2) was dissolved in 10 mL of methanol followed by addition of equimolar quantities of various substituted aldehydes or ketones with slow and constant stirring. Few drops of glacial acetic acid were added as an acid catalyst. The reaction mixture was refluxed at 40˚C for 4-6 h*. After the completion of the reaction, the solution was allowed to cool to room temperature. The solution was then poured into a petri dish and allowed to stay overnight for crystallization. The crystals were collected, washed with acetone and recrystallized with methanol.

* For Nitro and ketone compounds, the reaction was continued for 16 h to 18 h.

2-(3,5-dimethoxyphenylimino)methyl)phenol (**1**)

λ_{max} (nm): 266. IR (KBr, cm^{-1}): 1606 (C=N str); 3460 (Phenolic –OH str); 1141 & 1207 (aryl alkyl ether –CO str); 1573 (aromatic C=C str).^{1}H-NMR (300 Hz, δ H, $CDCl_3$): 3.83 (s, 6H, OCH_3); 6.40-7.40 (m, 7H, aromatic); 8.60 (s, 1H, N=CH); 13.14 (s, 1H, OH);. Anal. calcd (%) for $C_{15}H_{15}NO_3$: C 70.02, H 5.88, N 5.44; found (%): C 70.21, H 5.16, N 5.50.

N-(4-nitrobenzylidene)-3,5-dimethoxybenzenamine (**2**)

λ_{max} (nm): 267. IR (KBr, cm^{-1}): 1593 (-C=N str); 1518 (aromatic –C=C str); 1153 & 1205 (Nitro –NO str); 1468 (=C-H str).^{1}H-NMR (300 Hz, δ H, $CDCl_3$): 3.83 (s, 6H, OCH_3); 6.42 (m, 3H, aromatic); 7.60- 7.77 (m, 2H, aromatic-NO_2); 7.08 (d, *J*= 7.8Hz, 1H, aromatic-NO_2); 8.28 (d, *J* = 7.5 Hz, 1H, aromatic-NO_2); 8.93 (s, 1H, N=CH); Anal. calcd (%) for $C_{15}H_{14}N_2O_4$: C 62.93, H 4.93, N 9.79; found (%): C 62.89, H 4.86, N 9.77.

3,5-dimethoxy-N-((4-methoxyphenyl)(phenyl)methylene)benzenamine (**3**)

λ_{max} (nm): 316. IR (KBr, cm^{-1}): 1124 & 1153 (aryl alkyl ether –CO str); 1593 (C=N str); 1504 (aromatic –C=C str); 1325-1467 (aromatic multiple peaks of –CH str).^{1}H-NMR (300 Hz, δ H, $CDCl_3$): 3.74 (s, 9H, OCH_3); δ 6.39-7.98 (m, 12H, aromatic). Anal. calcd (%) for $C_{22}H_{21}NO_3$: C 76.06, H 6.09, N 4.03; found (%): C 76.20, H 6.03, N 4.14.

N-(3,4,5-trimethoxybenzylidene)-3,5-dimethoxybenzenamine (**4**)

λ_{max} (nm): 313. IR (KBr, cm^{-1}): 1591 (C=N str); 1124 & 1153 (aryl alkyl ether –CO str); 1504 (aromatic C=C str); 1487 (=C-H str).^{1}H-NMR (300 Hz, δ H, $CDCl_3$): 3.82 (s, 6H, OCH_3); 3.93 (s, 9H, OCH_3); 8.33 (s, 1H, N=CH); 6.30 (s, 3H, aromatic); 7.14 (s, 2H, aromatic). Anal. calcd (%) for $C_{18}H_{21}NO_5$: C 65.24, H 6.39, N 4.23; found (%): C 65.32, H 6.44, N 4.39.

N-(2-nitrobenzylidene)-3,5-dimethoxybenzenamine (**5**)

λ_{max} (nm): 270. IR (KBr, cm^{-1}): 1597 (-C=N str); 1521 (aromatic –C=C str); 1203 & 1344 (Nitro –NO str).^{1}H-NMR (300 Hz, δ H, $CDCl_3$): 3.79 (s, 6H, OCH_3); 6.33-8.21 (m, 7H, aromatic); 8.41 (s, 1H, N=CH). Anal. calcd (%) for $C_{15}H_{14}N_2O_4$: C 62.93, H 4.93, N 9.79; found (%): C 62.84, H 4.83, N 9.85.

4-(2-hydroxybenzylideneamino)benzoic acid (**6**)

λ_{max} (nm): 236. IR (KBr, cm^{-1}): 3423 (Phenolic –OH str); 3070-2856 (Carboxylic –OH str); 1689 (C=O str); 1610 (C=N str); 1572 (aromatic C=C str).^{1}H-NMR (300 Hz, δ H, DMSO): 5.86 (s, 1H, -OH); 7.53-7.73 (m, 8H, aromatic); 8.12 (s, 1H, N=CH); 12.01 (s, 1H, COOH). Elemental analysis, for, $C_{14}H_{11}NO_3$, calculated (%): C 69.70, H 4.60, N 5.81; found (%): C 70.51, H 4.42, N 6.19.

4-(benzylideneamino)benzoic acid (**7**)

λ_{max} (nm): 274. IR (KBr, cm^{-1}): 3066-2854 (Carboxylic -OH str); 1681 (C=O str); 1595 (C=N str); 1429 (aromatic C=C str).^{1}H-NMR (300 Hz, δ H, DMSO):; 7.52-8.12 (m, 9H, aromatic); 8.41 (s, 1H, N=CH); 12.00 (s, 1H, COOH). Elemental analysis, for, $C_{14}H_{11}NO_2$, calculated (%): C 74.65, H 4.92, N 6.22; found (%): C 74.36, H 4.97, N 6.90.

4-(2-nitrobenzylideneamino)benzoic acid (**8**)

λ_{max} (nm): 269. IR (KBr, cm^{-1}): 3408 (Carboxylic –OH str); 1703 (C=O str); 1624 (C=N str); 1348 & 1288 (Nitro –NO str); 1519 (aromatic C=C str).^{1}H-NMR (300 Hz, δ H, DMSO): 8.88 (s, 1H, N=CH); 7.33-8.12 (m, 8H, aromatic); 12.01 (s, 1H, COOH). Elemental analysis, for, $C_{14}H_{10}N_2O_4$, calculated (%): C 62.22, H 3.73, N 10.37; found (%): C 64.90, H 4.04, N 9.65.

4-(4-nitrobenzylideneamino)benzoic acid (**9**)

λ_{max} (nm): 277 . IR (KBr, cm^{-1}): 3383 (Carboxylic –OH str); 1683 (C=O str); 1600 (C=N str); 1521 (aromatic C=C str); 1342 & 1294 (Nitro -NO str). ^{1}H-NMR (300 Hz, δ H, DMSO): 7.52-8.25 (m, 8H, aromatic); 8.84 (s, 1H, N=CH); 12.10 (s, 1H, COOH). Elemental analysis, for, $C_{14}H_{10}N_2O_4$, calculated (%): C 62.22, H 3.73, N 10.37; found (%): C 63.46, H 3.82, N 10.32.

4-(3,4,5-trimethoxybenzylideneamino)benzoic acid (**10**)

λ_{max} (nm): 280. IR (KBr, cm^{-1}): 3070-2881 (Carboxylic –OH str); 1681 (C=O str); 1597 (C=N str); 1579 (aromatic C=C str); 1421 (-OH bending); 1286 & 1126 (aryl alkyl ether –CO str).^{1}H-NMR (300 Hz, δ H, DMSO): 3.71 (s, 9H, OCH_3); 6.61-8.15 (m, 6H, aromatic); 8.42 (s, 1H, N=CH); 12.0 (s, 1H, COOH). Elemental analysis, for, $C_{17}H_{17}NO_5$, calculated (%): C 64.75, H 5.43, N 4.44; found (%): C 65.05, H 5.41, N 4.25.

4-((4-methoxyphenyl)(phenyl)methyleneamino)benzoic acid (**11**)

λ_{max} (nm): 285. IR (KBr, cm^{-1}): 2924-2777 (Carboxylic –OH str); 1662 (C=O str); 1600 (C=N str); 1174 & 1238 (Ether C-O str); 1319 (Carboxylic C-O str); 1495 (aromatic C=C str).^{1}H-NMR (300 Hz, δ H, DMSO): 3.85 (s, 3H, -OCH_3); 5.85 (s, 2H, aromatic); 6.53 (d, *J* = 8.1, 2H, aromatic); 7.08 (s, *J* = 8.4, 2H, aromatic); 7.53-7.75 (m, 7H, aromatic); 11.96 (s, 1H, -COOH). Elemental analysis, for, $C_{21}H_{17}NO_3$ calculated (%): C 76.12, N 5.17, H 4.23; found (%) C 76.73, H 5.76, N 4.99.

4-(1-(4-hydroxy-3-methoxyphenyl)ethylideneamino)benzoic acid (**12**)

λ_{max} (nm): 278. IR (KBr, cm^{-1}): 3363 (Phenolic –OH str); 2847 (Carboxylic –OH str); 1662 (C=O str); 1578 (C=N str); 1602 (aromatic C=C str); 1209 and 1168 (aryl alkyl ether –CO str).^{1}H-NMR (300 Hz, δ H, DMSO): 3.33 (s, 3H, -CH_3); 3.84 (s, 3H, -OCH_3); 5.86 (s, 2H, aromatic); 6.53 (d, *J* = 8.7, 2H, aromatic); 6.86 (s, *J* = 8.1, 1H, -OH); 7.43-7.62 (m, 3H, aromatic); 11.93 (s, 1H, -COOH). Elemental analysis, for,$C_{16}H_{15}NO_4$, calculated (%): C 66.41, H 4.83, N 5.16; found (%): C 65.12, H 4.20, N 5.55.

4.3. Estimation of cholinesterase activity (in-vitro)

The effectiveness of the tested compounds to inhibit acetylcholinesterase from the electric eel (E.C. 3.1.1.7) and butyrylcholinesterase from human serum (E.C. 3.1.1.8) was examined with IC_{50} values. Ellman's spectrophotometric analysis[35] was used to determine IC_{50} values by recording the rate of increase in the absorbance at 412 nm for 5 min. A stock solution of AChE was prepared by dissolving AChE in 0.1 M phosphate buffer (pH 8.0), and a stock solution of BChE was prepared by dissolving the lyophilized powder in an aqueous solution of gelatin 0.1%. The final assay solution consisted of 0.1

M phosphate buffer (pH 8.0) with 340 mM 5,5'-dithiobis(2-nitrobenzoic acid), 0.02 unit/ml of AChE or BChE and 550 mM of substrate (acetylthiocholine iodide, ATCh or butyrylthiocholine iodide, BTCh, respectively). Different concentrations of test compounds between 20 and 80% were selected to obtain inhibition of the enzymatic activity. From aliquots (50 µL), increasing concentrations of the compounds were added to the assay solution after preincubation for 20 min at 37 °C with the enzyme followed by the addition of substrate. The blank assay consisted of all components except AChE or BChE to account for the non-enzymatic reaction. The reaction rates were compared, and the percent inhibition due to the increasing concentrations of the compound was calculated. The concentration of each test compound was recorded in triplicate, and their IC_{50} values were determined graphically from percent inhibition curves.[36a, 36b]

4.4. Enzyme kinetics study

Ellman's spectrophotometric analysis was used to identify the type of inhibition. ATCh and BTCh were used as substrates in different concentrations, both below and above Km, in an alternative sequence in a phosphate buffer at pH 8, with a fixed concentration of cholinesterase, in the absence or presence of different compounds. The compound concentration was maintained close to the IC_{50} value of enzyme inhibition. The inhibitory kinetics were evaluated by the Lineweaver and Burk method.[37]

4.5. Free radical scavenging activity

4.5.1. DPPH (2, 2-diphenyl -1-picryl -hydrazyl) radical scavenging activity

The free radical scavenging activity of the synthesized compound 4 was examined using DPPH (*2, 2-diphenyl -1-picryl -hydrazyl*) method.[30] Ascorbic acid ((5R)-[(1S)-1,2-Dihydroxyethyl]-3,4-dihydroxyfuran-2(5H)-one) was used as standard. 1 ml solution of each compound at different concentration (10, 100, and 500 µg/ml) in methanol was mixed with 2 ml of DPPH solution (0.5 mM). The mixture was shaken vigorously and allowed to stand in dark place at room temperature for 30 min, and the absorbance was read at 517 nm. The antiradical activity was calculated in percentage as per the following formula: Free radical scavenging activity (%) = (absorbance of control – absorbance sample/absorbance of control) × 100.

4.5.2. Hydrogen peroxide radical scavenging activity

The scavenging ability of the hydrogen peroxide radical of the compound 4 was measured by H_2O_2 radical scavenging assay.[31] A solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer saline (pH-7.4) at 20ºC. Hydrogen peroxide solution (0.6mL) was added to 1mL of standard ascorbic acid (10 µg/mL) and different concentration of compound **4** (10, 100 and 500 µg/mL). The absorbance of hydrogen peroxide was determined at 230 nm in UV/visible spectrophotometer after 10 minutes against a blank solution containing phosphate buffer saline. The assay was performed in triplicate. The hydrogen peroxide radical scavenging activity was measured by the following equation. H_2O_2 activity (%) = (Absorbance of control - Absorbance of sample / Absorbance of control) × 100.

4.6. Molecular docking

4.6.1. Preparation of the protein

The BChE complexed with N- [(3R)-1-(2,3-dihydro-1H-inden-2-yl)piperidin-3-yl]methyl-N-(2methoxyethyl)naphthalene- 2-carboxamide (3F9) (PDB Code: 4TPK) , and AChE complexed with E2020 (donepezil) (PDB Code: 1EVE) was chosen as the target protein.[38] The structures were prepared using the protein preparation wizard in Maestro 10.5.014. by adding hydrogen, assigning partial charges using the OPLS-2005 force field, assigning protonation states, and determining restrained and partial energy minimization. The ligand was removed, and the ligand binding site was defined. The default settings were used for all other parameters. The extra precision (GLIDE-XP) implemented in GLIDE was used for docking.

4.6.2. Molecular docking

The crystallographic and trajectory water molecules, ions and ligand compounds were removed from developed protein. The ligands were built using Maestro 10.5.014 build panel and prepared by Lig Prep 3.9 (Schrödinger, LLC, USA, 2016-1) application that uses OPLS 2005 force field. OPLS stands for optimized potential liquid simulations, and it gave the corresponding energy minima 3D conformers of the ligands. The proteins were prepared using Schrodinger software, Maestro, and Glide. The Glide XP algorithm was employed, using a grid box volume of 10_10_10 Å. All the structures were fitted into the binding pocket, and the lowest energy pose for each docking run was retained.[39]

4.7. Determination of partition coefficient

The lipophilic constant of all of the compounds (**1-12**) was determined in n-octanol and buffer (pH 7.4) by the shake flask method[40]. The log P was calculated by correlating the absorbance with the concentration using a standard plot.

4.8. Determination ADME properties using Qikprop

The ADME properties were assessed for the ligands obtained from LigPrep. LigPrep generated ligand with the desired structural and chemical features for further computational analyses. The compounds (**1-12**) along with donepezil were subjected to QikProp module of the Schrödinger suite to calculate the ADME properties. The newly identified ligands checked for octanol/water partition coefficient, Lipinski's rule of five and Jorgensen's rule of three, gut-blood barrier, binding to human serum albumin, aqueous solubility, brain/blood partition coefficient, skin permeability, and percent of human oral absorption.

4.9. Biological studies

4.9.1. Animals

Charles Foster rats of the albino strain (4 to 5 months old and 150 to 200 g) of either sex were procured from the Central Animal House, Institute of Medical Sciences, Banaras Hindu University, Varanasi. They had access to water *adlibitum* and were fed a semi-synthetic balanced diet, with an occasional supply of green vegetables (salad leaves). Six rats were housed per cage at 22 ºC ± 3 ºC and 45-55% relative humidity. Twelve-hour of light and dark cycles was strictly followed in a fully ventilated room. Prior permission was obtained from the Institutional Animal Ethical Committee (Registration No: 542/02/ab/CPCSEA).

4.9.2. Ex vivo AChE inhibition

The *ex vivo* assay was performed to assess the AChE inhibition of compound **4**. Test drug was given orally (p.o.) to rats at doses of 10 mg/kg and 30 mg/kg. After one hour, the animals were sacrificed by decapitation and the brain was instantly dissected, cleaned with cold saline and kept in

phosphate buffer (pH 8.0). Entire brain was then homogenate and centrifuged to get the supernatant. The collected supernatant was used for assay following Ellman method to calculate the percentage of brain AChE Inhibition.

4.9.3. Drug treatment

The rats were divided into 5 groups with each group having 6 rats. Group 1 was considered as control whereas 2, 3, and 4 were the test, and group 5 was standard. The compound **4** was administered as an oral dose of 10, 20 and 30mg/Kg in 0.3% CMC suspension respectively in the test groups. Donepezil was administered as an oral dose of 10mg/Kg in the standard group.[41] The treatment schedule is mentioned in the supplementary table 2.

4.9.4. Morris water maze (MWM) task

The Morris water maze is a fabricated metal semi-circular pool (120 cm in diameter, 45 cm deep) filled with water to a depth of approximately 25 cm. The pool was divided into four equal quadrants, and a platform (15 cm in diameter) was submerged 0.5 cm below the opaque surface in the center of one of the quadrants. The pool was located in the center of a small test room and was surrounded by many extra-maze cues on the walls of the room and two on the rim of the pool to provide both proximal and distal visual cues. Individual rats from all groups were placed by hand into the water facing the wall of the pool and were then given 60 sec to find the hidden platform. When successful, the rat was then given 20 sec on the platform to watch the spatial cues. If the animal failed to find the platform in this time, it was guided there. Escape latency, swim distance and time spent in the target quadrant were scored by ANY- Maze video tracking software.[42,43]

4.9.4.1. Habituation

All the rats used in the study were habituated to the pool by allowing them to perform a 60 sec swim without the platform 24 h before starting the training.

4.9.4.2. SRM testing in the water maze

SRM testing began on day 7 of treatment, 1 h after the last dose of a compound or donepezil. The animals underwent eight trials/day (separated by 10 min) for three consecutive days (days 7–9). In each trial, the animal was released from a different starting point in the pool, but the escape platform was kept in the same position. Each start location was located in the middle of a quadrant at the edge of the pool. After three days of training (days 7–9), the animals were returned to their home cages until the retention testing (probe trial) 24 h later on day 10. The probe trial consisted of a 60 sec free swim period without a platform, in which the time spent in the target quadrant was recorded by ANY- Maze video tracking software.

4.9.4.3. SWM testing in the water maze

Rats received eight trials per day in the reference memory test. This initial testing prepared the animals for the working memory task.[44] After 48 h of the reference memory pre-training phase on day 13, the working memory task began. Two trials per day were given over four consecutive days (days 13–16). In the first trial (acquisition), the rat had to find the platform in a new position (new task) each day. On the second trial (retrieval), which was performed 75 min later, the platform was in its previous position, but the animal started from one of three possible locations different from the preceding trial.

4.9.5. In-vivo activity of GSH estimation.

4.9.5.1. Drug Treatment

The rats were divided into 4 groups with each group having 3 rats. Group 1 was considered as normal control saline whereas group 2 was positive control and treated with aspartame orally at dose level of (75 mg/kg.weight.body),[33] group 3 was treated with aspartame plus compound **4** (10 mg/kg.weight.body) and group **4** was treated with aspartame plus donepezil (10 mg/kg.weight.body). Brain Sample was obtained as per *ex vivo* method (Section 4.9.2.).

4.9.5.2.. GSH estimation

Estimation of glutathione (GSH) in the brain tissue homogenate was performed by the method of Ellman, using 5,5'-Disulfanediylbis(2-nitrobenzoic acid) (DTNB). In this method when DTNB was added to the sample containing sulfhydryl groups that give yellow color and estimated at 412 nm. The concentration of GSH was calculated from standard GSH curve and expressed in µmol/mg of protein.

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Supplementary Material

Detailed experimental procedures with analytical data for compounds including ^{1}H NMR. (PDF)

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